Structural mechanisms of Long-term potentiation in the dentate gyrus of freely moving rats

Ryan Ellis

Supervised by Jared B Bowden & Kristen M Harris

Center for Learning and Memory, Dept. of Neurobiology, The University of Texas at Austin.

A thesis presented to The University of Texas at Austin for partial completion of Special Departmental Honors in Biology

Signatures of Approval:

Supervising Professor:

_____

Biology Honors Advisor:

_____

Abstract

Long-lasting enhancement of synaptic transmission, known as long-term potentiation (LTP), is involved in learning and memory and is associated with the function of tiny dendritic protrusions called spines. Importantly, our understanding of how these structures change to allow for the expression of LTP is incomplete. Previous studies conducted in the dentate gyrus (DG) have demonstrated that LTP is associated with increases in spine volume and synaptic area; however, controversy remains as to whether these changes are accompanied by alterations in the total number of spines on the dendritic shaft. Here, we have used *in vivo* electrophysiology, serial section electron microscopy, and a novel method of unbiased sampling to investigate how spines, synapses, and cellular organelles function to allow for the expression of LTP in the dentate gyrus of freely moving rats. We hypothesize that LTP would not be associated with significant changes in the density of spines along the dendritic shaft, but rather, expressed through increases in the volume and synaptic area of existing spines. Research has shown that an organelle known as the spine apparatus is involved with enlarging synapses. We hypothesized that spines containing a spine apparatus (SA) would show the largest increases in volume and synaptic area. LTP was induced by applying delta-burst stimulation to the medial perforant path projection to the middle molecular layer (MML) of the dentate gyrus of 2 adult, male rats. Thirty-minutes after the induction, the MML was processed for electron microscopy (EM) and the images were uploaded into Reconstruct, a software tool developed for the three-dimensional analysis of neuronal structures. Our results demonstrated that LTP was not associated with significant changes in spine density. Subsequent analysis revealed that average spine volume increased significantly 30 minutes following LTP. Marginal increases were also observed in average synapse area, however, this change did not reach significance. Overall, we found that the largest increases in spine volume and synaptic area were found on spines containing a SA. These effects were highly significant, and emphasize the importance of the spine apparatus in cellular models of learning and memory.

Collaborators

Larry Lindsey, John Mendenhall, Masaaki Kuwajima, Amy Pohodich, Michael Musslewhite, Junho Ahn, Patrick Parker

Center for Learning and Memory, Dept. of Neurobiology, The University of Texas at Austin

Introduction

The neurons of the brain communicate across electrochemical junctions known as synapses; 90% of these excitatory synapses exist on small protrusions called dendritic spines. Electrophysiological studies have demonstrated synaptic transmission and can undergo persistent increases in efficacy, known as long-term potentiation (LTP); however, our understanding of this phenomenon and how it relates to structure remains incomplete. The aim of this investigation was to understand how synapses and dendritic spines change with LTP, and to identify the cellular organelles that could support these changes.

The dentate gyrus (DG) is a subregion within the mammalian hippocampus that has been implicated in the processes of learning and memory (Douglas and Goddard, 1975; Abraham and Goddard, 1983; Doryére et al., 1997; Treves et al., 2008). This structure is an advantageous model system to study the structural correlates of LTP for two reasons. First, previous studies have shown that the dentate gyrus is capable of supporting highly robust and persistent LTP. Abraham et al (2002) demonstrated that the this stable form of LTP can last for many months. Second, the uniform nature of the perforant path input to the dentate gyrus means that LTP can be induced within the spatially specific region or middle molecular layer of the dentate granule cell dendritic tree and easily excised for structural analysis (Bowden, 2011). In the following investigation we have used *in vivo* electrophysiology, serial section electron microscopy, and quantitative 3D reconstruction to investigate how spines, synapses, and dendritic organelles change 30 minutes following the induction of LTP.

While LTP is well understood electrophysiologically, the structural correlates of LTP are still under debate. Previous studies conducted in hippocampal area CA1 have shown that long-term synaptic plasticity is associated with a range of alterations involving dendritic spines (Engert and Bonhoeffer, 1999; Bourne and Harris, 2007). These changes involve increases in individual spine size, changes in spine shape-and-type, the movement of cellular organelles such as polyribosomes, or changes in the number of spines along the dendritic shaft (Popov et al., 2004; Bourne and Harris, 2011, 2007; Desmond and Levy, 1983). Studies performed in the DG have shown particularly inconsistent results with regards to changes in the density of spines on the dendritic shaft after the induction of LTP. Desmond and Levy (1983) found that LTP was associated with increases in spine density, whereas Geinisman et al. (1991) found that LTP was associated with decreases in spine

density. In 2004, Kirov et al. showed no net change in synaptic density after induction of LTP. We believe that these conflicting reports could be attributable to the methods used to select dendritic segments for sampling. Recent work in the Harris lab has demonstrated that microtubule content of a dendritic segment is highly correlated with its respective spine density. By matching our dendritic samples for microtubule content we can avoid inherent difference in spine density, and ensure that our data is based valid dendritic comparisons. We refer to this sampling technique as *unbiased sampling*. We hypothesize that LTP in the dentate gyrus will not be associated with significant changes in spine density.

A previous study performed in the Harris lab in hippocampal area CA1 showed a relationship between LTP and increases in spine size and shape (Bourne and Harris, 2007). Modeling studies have found that these morphological changes could be sufficient to explain observed increases in synaptic transmission (Yuste and Bonhoeffer, 2001; Nusser et al., 1998). The relationship between long-term potentiation and increases in average spine volume may be explained as large spines typically contain a greater number of postsynaptic signalling molecules, and increases in the number of signalling molecules results in the strengthening of synaptic transmission (LTP). In addition to this, larger spines may have a greater number of signalling molecules. These increases in synaptic signaling molecules can be measured within our experiment as increases in synaptic area. We hypothesize that LTP in the dentate gyrus will be associated with significant increases in spine volume and synaptic area.

The spine apparatus is an organelle present in more than 80% of the largest spines in the hippocampus; interestingly, its role in the induction of LTP remains largely unknown (Bourne and Harris, 2007). The SA is an extension of the endoplasmic reticulum network and animals lacking spine apparatuses show deficits in LTP as well as the actin-binding protein, synaptopodin (Spacek and Harris, 1997; Jedlicka et al., 2008; Deller et al., 2003). Synaptopodin (SP) is localized within the spine apparatus and is used in the formation of the postsynaptic density (Jedlicka et al., 2008). We hypothesized that spines containing SA would show the largest increases in spine volume and synaptic area.

It was found that the structural changes 30-minutes after the induction of LTP in the DG, spine density did not experience a significant change. Increases in spine volume and synaptic area were also observed, the biggest of which were in the spines containing a SA.

Materials and Methods

Animals, Surgery, Electrophysiology

Two adult (150 days old, 400-700 gm) male Long-Evans rats were anesthetized (ketamine, 75 mg/kg s.c.; domitor, 0.5 mg/kg s.c.), placed in a stereotaxic apparatus, and implanted with two monopolar stimulating electrodes and one monopolar recording electrode. The stimulating electrodes were placed in the medial and lateral perforant path fibers of the left (experimental) hemispheres and just medial perforant path fibers in the right (control) hemispheres. The recording electrode was placed in the dentate hilus in both hemispheres (see Fig. 1). Lateral perforant path recordings were used for another study and will not be further discussed. Once electrode positions were finalized, they were secured to the skull with an acrylic resin. After the completion of the surgical procedures, the

rats were closely monitored during a 2-week recovery period prior to beginning the electrophysiological recordings.

Following recovery, animals were tested for viable recordings. Separation testing was used to determine if the electrodes were stimulating different pathways. Convergence testing was used to determine whether or not the stimulating electrodes were activating a common postsynaptic target (McNaughton and Barnes, 1977; Bowden 2010). Animals that met the criteria of these tests were used for baseline recording experiments.

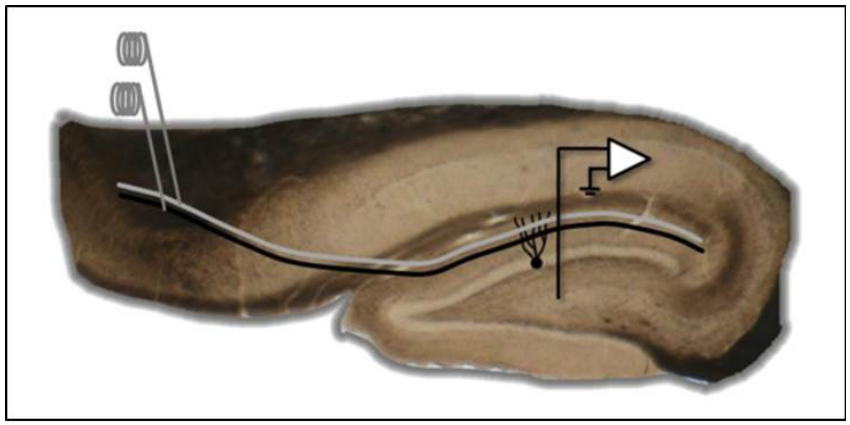

Figure 1: Illustration showing electrode positions. The stimulating electrode is implanted in the medial perforant path (left – black line) and the lateral perforant path (left – grey line). fEPSPs evoked by activation were recorded by an electrode implanted in the dentate hilus (right).

Field excitatory postsynaptic potentials (fEPSPs) were used as a measure of synaptic transmission and DBS has been shown to induce persistent increases in the fEPSP slope. This state is referred to as long-term potentiation (Bliss and Lomo, 1973; Douglas and Goddard, 1983; Bowden, 2010). One day following the fEPSP baseline stability was confirmed, a delta burst stimulation (DBS) protocol was performed in the experimental hemisphere while baseline slopes were maintained in the control hemisphere (see Fig. 2). All animals were deeply anaesthetized and perfused 30 minutes after the stimulation ended.

All of the surgical and electrophysiological procedures were performed as part of a previous experiment at the University of Otago as previously described in Bowden, 2011. These methods were approved by the University of Otago Animal Ethics Committee and complied with NIH requirements for the humane care and use of laboratory animals.

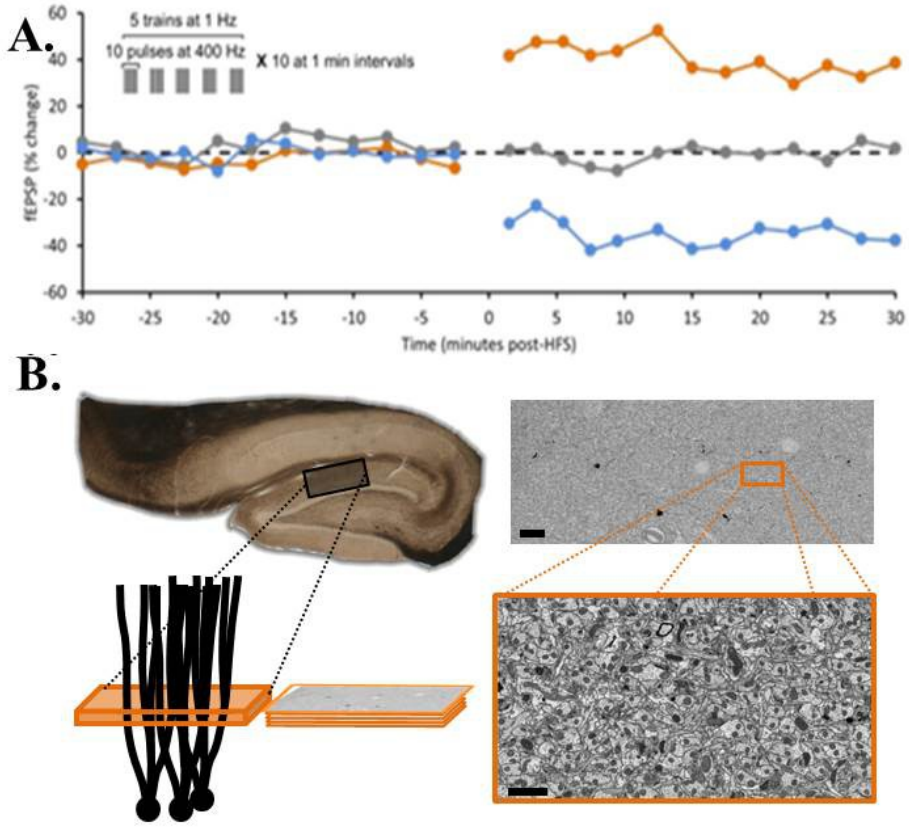

Figure 2: A. Electrophysiology from an animal used in this study (400HzDBS). Orange plot is LTP data, grey plot is control, blue plot wasn't used for this experiment. **B.** Tissue was processed for electron microscopy, ultrasectioned (45nm thick sections) into series (~200 sections) and imaged with a transmission-mode scanning electron microscope. Scale bars: 18 µm (top), 2 µm (bottom).

Tissue perfusion-fixation

Animals were deeply anesthetized with isoflurane (5%) and transcardially perfused with an oxygenated Krebs-Ringer Carbicarb buffer for 15 seconds, and chemical fixative (2.5% glutaraldehyde, 2% paraformaldehyde, 2 mM $CaCl_2$, and 4 mM $MgSO_4$) for 2 hours, in 0.1 M cacodylate buffer, pH 7.35-7.4.

Preparation for electron microscopy

The fixed brains were removed and vibrasliced to ~70nm (Leica VT 1000S, Leica, Nusslock, Germany), then rinsed with the 0.1M cacodylate buffer. Tissue containing the dentate gyrus was removed and embedded in 7% agarose, treated with 1% osmium and 1.5% potassium ferrocyanide in

100 mM cacodylate buffer, and rinsed in buffer before being immersed in 1% osmium and microwaved, cooled to 20°C, and rinsed in buffer.

Embedded tissue blocks containing either the control or LTP tissue were then ultrasectioned into ~200 consecutive serial sections of ~45nm thickness.

ssTEM imaging

Each series was placed in a rotating cassette and photographed with a JEOL 1200EX (Peabody, MA) transmission electron microscope. Images were then aligned and uploaded to Reconstruct software designed for the use of the Harris Lab.

Measured variables

Microtubule number was calculated as an average from 3 samples taken across the ~200 sections. Microtubule content would be used in the unbiased sampling of dendrites.

Analyzed length in the z-dimension (z-length) of each dendrite was measured throughout the extent of its appearance through the series from the first complete spine to the first incomplete (goes out of image) spine. Spine density was calculated from the total number of spines divided by the z-length.

Spine volumes and postsynaptic density (PSD) areas were manually measured using functions available in the Reconstruct program. Dimensions were initially measured on the sections on which they appeared then 3-dimensionally reconstructed to give the volume.

Each spine apparatus found in a dendritic spine, defined as being 0.1 microns from the base of the spine, was stamped and labeled according to its associated spine to be used for later analysis.

We chose dendrites whose microtubule content fell within the average for the MML which was between 30-35 microtubules.

All spine volume, density, and synaptic surface area data was compared across experimental conditions with an analysis of variance (ANOVA). An alpha level of 0.05 was defined as statistically significant for all analyses.

Results

For this study, Long-Evans rats underwent the induction of LTP in the dentate gyrus and the control hemispheres received baseline stimulation.

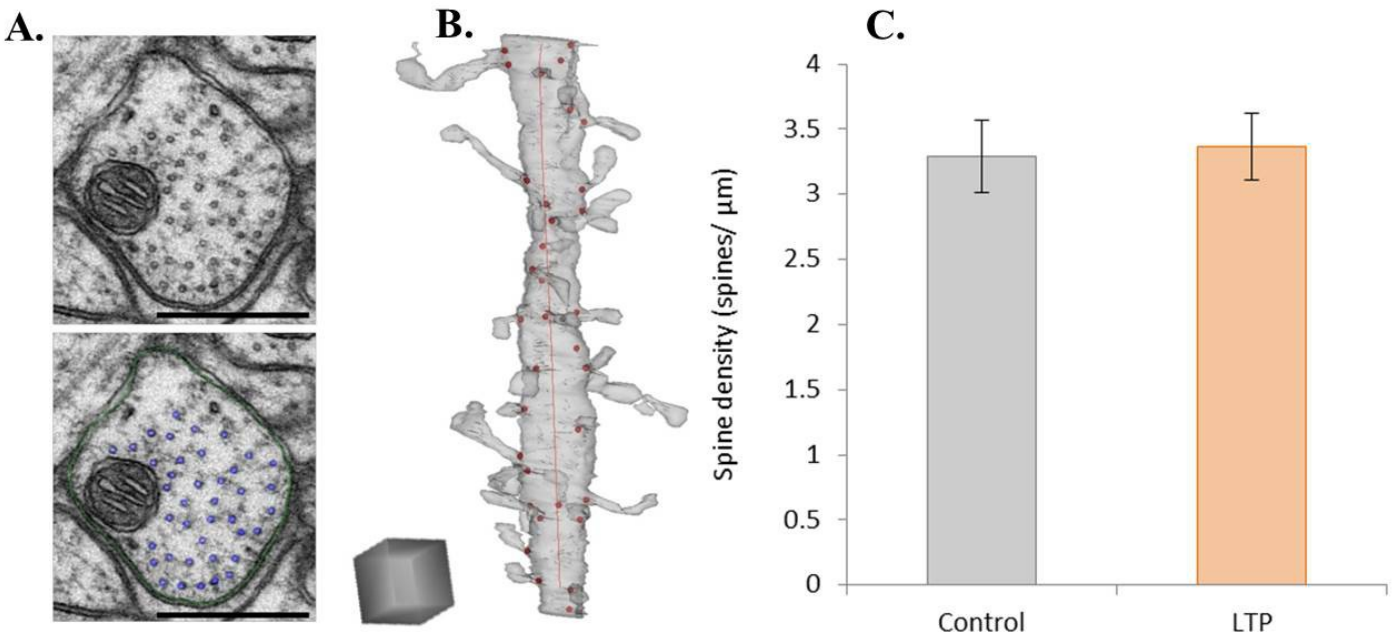

Figure 3: A. Quantification of microtubule number. Each microtubule was stamped as shown on three sections per dendrite, scale bar = 0.5 µm^3 **B.** Quantification of spine density. A reconstruction of a dendrite shows the z-length trace down the shaft and stamps marking each spine, scale cube = 1 µm^3. **C.** Analysis showed no significant increase in spine density.

Statistics performed with an ANOVA revealed no significant difference in the density of dendritic spines between LTP and control conditions ($F_{(1,20)} = 0.038$, $P = 0.846$; Fig. 3C). Marginal changes amounted to no more than a 2.3% increase in the density of spine following LTP.

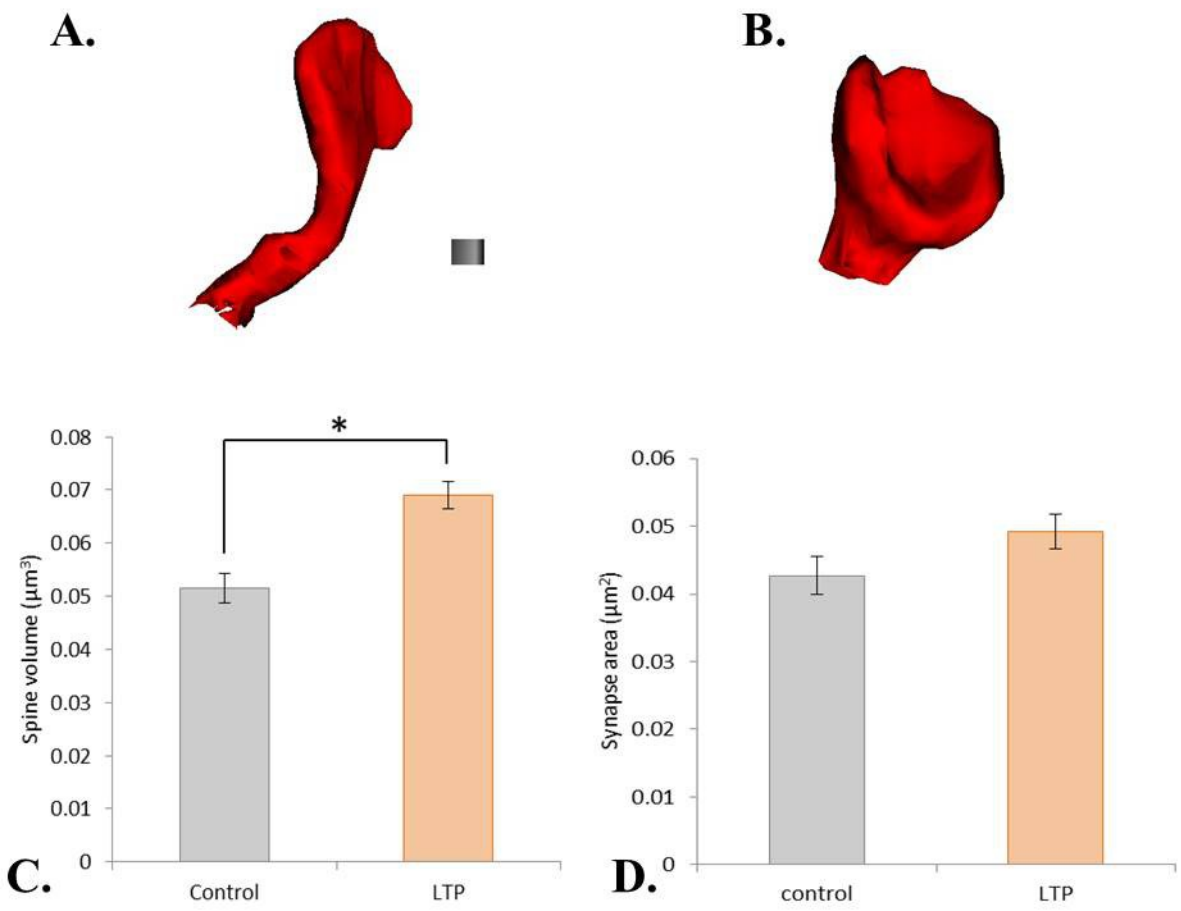

Figure 4: A. Reconstruction of a spine from the control hemisphere, scale cube = 0.1 μm^3. **B.** Reconstruction of a spine from the LTP hemisphere. **C.** A significant increase in spine volume is shown **D.** A trending, yet not significant increase in synapse area is shown.

Significant increases (34.3%) in spine volume were observed 30 minutes following the induction of LTP ($F_{(1,640)}$ = 10.790, P = 0.001; Fig.4C). Average synaptic surface area was found to be 15.1% higher than baseline, 30 minutes following LTP; however, this increase did not reach statistical significance ($F_{(1,640)}$ = 2.896, P = 0.089; Fig.4D).

We then conducted analysis to determine whether increases in spine volume and synapse area were largest on spines that contained a spine apparatus. Our results demonstrated that increases in spine volume associated with LTP were most pronounced on spines that contained a spine apparatus (43.3%, $F_{(1,66)}$ = 6.560, P = 0.012; Fig.5C). In contrast with the trending increases in synaptic area seen previously, our results indicated that synaptic area increases were significantly larger and therefore also associated with spines containing a spine apparatus (42.9%, $F_{(1,66)}$ = 7.297, P = 0.008; Fig.5D).

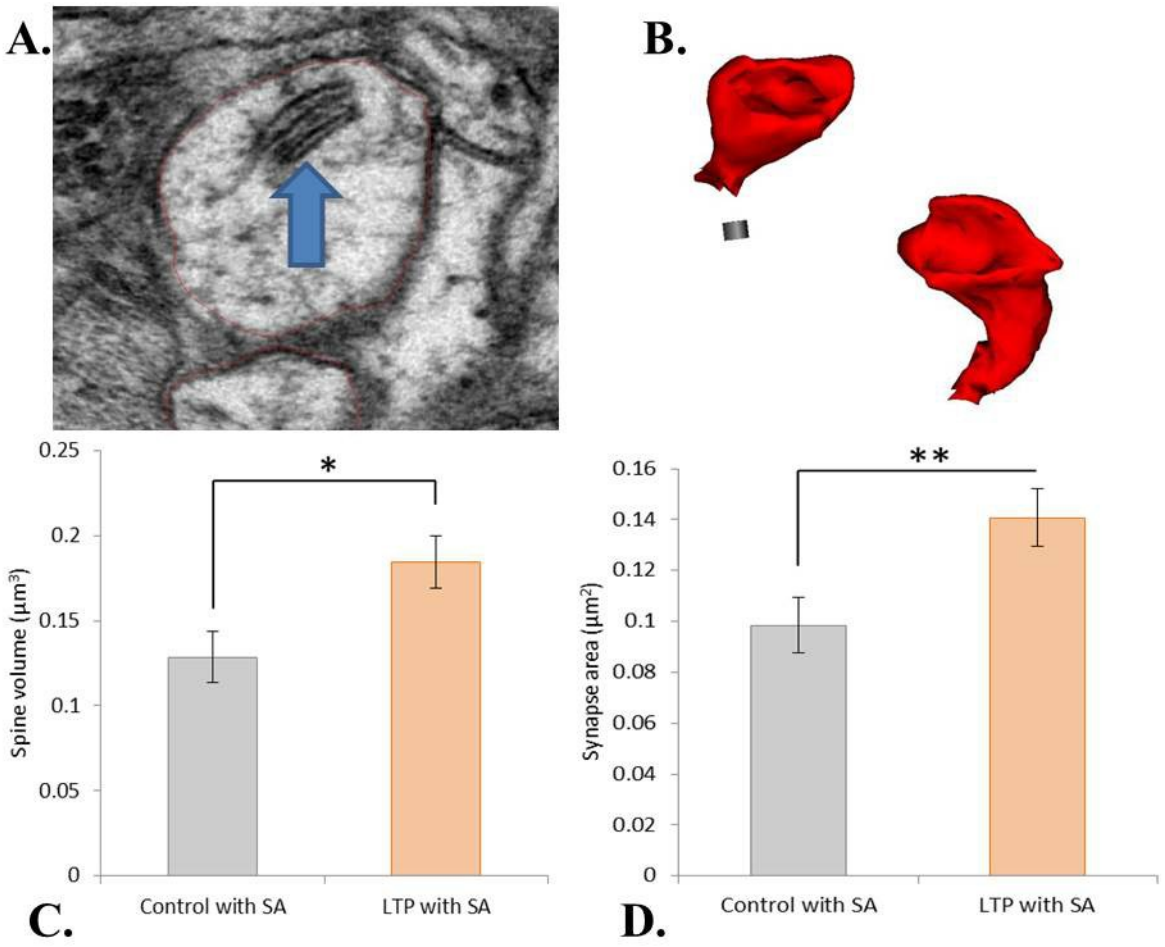

Figure 5: A. An electron micrograph showing a spine apparatus in a spine, indicated by blue arrow. **B.** Reconstructions of a control spine (top left) and a spine from the LTP hemisphere (bottom right). Both contained spine apparatuses, scale cube = 0.1 µm³. **C.** A significant increase in spine volume is shown. **D.** A significant increase in synapse area is shown.

Discussion

The aim of this investigation was to identify the structural correlates of long-term enhancement in synaptic transmission. *In vivo* electrophysiology followed by electron microscopy, unbiased sampling method, quantitative three-dimensional reconstruction, and statistical analysis led us to determine that the enhancement in synaptic efficacy is associated with an increase in size of synaptic connections and not an increase in the number of synaptic connections. We saw no change in the spine density 30-minutes after the induction of LTP. Changes in the size of the synaptic connections occurred with LTP as increases in spine volume and synaptic surface area were seen in the experimental hemispheres. Spines containing SAs proved to be the most changed after LTP compared to control with increases of ~43% in both size and synaptic area.

Previous studies focused on the association between LTP and spine density have yielded conflicting results (Desmond and Levy, 1983; Geinisman et al., 1991; Kirov et al., 2004). Our understanding of the relationship between microtubule content and spine density allows us to choose and compare dendrites with similar spine densities preventing us from effectively comparing “apples to oranges”. By using this unbiased sample method we found that LTP resulted in no net change in spine density and have a high degree of confidence in our finding due to this unbiased sampling. Since this study is specific to only the DG under conditions of LTP, our conclusions remain limited to this region as we cannot speculate whether or not changes in spine density may be occurring elsewhere in the brain. Spine density may play role in the homeostatic maintenance of synaptic activity. A previous study stated that LTP inducing stimulation has little or no net effect in the total number of spines and synapses (Kirov et al., 2004). However, they explain that abnormal, epileptic activity is associated with substantial spine loss (Kirov et al., 2004). Inversely, if synaptic transmission is blocked or critically reduced, spine proliferation is initiated (Kirov et al., 2004). Therefore, the number of spines could be used to maintain a “set level” of synaptic activity rather than involved with changes induced by patterns of LTP inducing stimulation.

Both average spine volume and synaptic area increased after the induction of LTP, but the largest increases were associated with spines containing a spine apparatus. This finding leaves us speculating the spine apparatuses role in the long-term enhancement of synaptic transmission. Research has shown close association of the spine apparatus with synaptopodin, an actin-associated protein involved in cell motility (Jedlicka et al., 2009). Actin is used for spine shape and the construction of the postsynaptic density (Fischer et al.,1998). Furthermore, synaptopodin-deficient mice result in the impaired induction of LTP and necessary for the formation of the spine apparatus, an extension of endoplasmic reticulum (Mundel et al., 1997; Deller et al., 2003). Since LTP is a synapse specific event and not all spines undergo potentiation, spines receiving the synaptic activity will require the structural components necessary for the increase in volume and PSD area (Isaac et al., 1995). Synaptopodin-deficient mice have dendritic spines completely void of spine apparatuses, therefore, studying the induction of LTP in synaptopodin knockout mice could clarify the role a spine apparatus plays in changing the structure of spines (Deller et al., 2003).

Finally, this study can be extended to other regions of the DG such as the outer molecular layer and inner molecular layer which undergo a concurrent form of long-term depression (cLTD) when LTP is induced in the MML. cLTD is expressed as a long-lasting decrease in synaptic transmission; operationally conceptualized as the the converse of LTP. As such, we would hypothesize that cLTD in the inner and outer molecular layers of the dentate gyrus would be associated with decreases in spine volume. As found in this study, we do not expect that these changes will be accompanied by overall increases or decreases in spine density. It is unclear how the presence-or-absence of a spine apparatus would affect the expression of cLTD in the dentate gyrus; an investigation of these organelles and possible associations would be an interesting future direction for this research.

In conclusion, the result of this study demonstrated that LTP in the dentate gyrus is not associated with changes in the density of spines on the dendritic shaft, but rather, an overall increase in the volume of dendritic spines, as well as marginal increases in synaptic surface area. These increases are closely related with the presence of the spine apparatus, an organelle known to have a key role the induction of LTP. With this result, we are left with a broader understanding of the role spine apparatuses play in the modification of dendritic spines to allow for the induction of LTP.

References

Abraham WC, Mason-Parker SE, Bear MF, Webb S, Tate WP (2001) Heterosynaptic metaplasticity in the hippocampus in vivo: a BCM-like modifiable threshold for LTP. Proceedings of the National Academy of Sciences of the United States of America 98:10924–10929.

Abraham WC, Christie BR, Logan B, Lawlor P, Dragunow M (1994) Immediate early gene expression associated with the persistence of heterosynaptic long-term depression in the hippocampus. Proceedings of the National Academy of Sciences of the United States of America 91:10049–10053.

Abraham WC, Goddard G (1983) Asymmetric relationships between homosynaptic long-term potentiation and heterosynaptic long-term depression. Nature 305:717–719.

Abraham WC, Logan B, Greenwood JM, Dragunow M (2002) Induction and experience-dependent consolidation of stable long-term potentiation lasting months in the hippocampus. The Journal of Neuroscience 22:9626–9634.

Bliss TVP, Lomo T (1973) Long-Lasting Potentiation Of Synaptic Transmission in the Dentate Area of the Anaesthetized Rabbit Following Stimulation of the Perforant Path. J Physiol:331–356.

Bourne J, Harris KM (2007) Do thin spines learn to be mushroom spines that remember? Current opinion in neurobiology 17:381–386.

Bourne JN, Harris KM (2011) Coordination of size and number of excitatory and inhibitory synapses results in a balanced structural plasticity along mature hippocampal CA1 dendrites during LTP. Hippocampus 21:354–373.

Bowden JB (2010) Structural Mechanisms of Bidirectional Synaptic Plasticity in the Dentate Gyrus of Freely Behaving Rats.

Bowden JB, Abraham WC, Harris KM (2011) Differential effects of strain, circadian cycle, and stimulation pattern on LTP and concurrent LTD in the dentate gyrus of freely moving rats. Hippocampus 000.

Bowden JB, Mendenhall JM, Abraham WC, Harris KM. (2008). Microtubule number as a correlate of dendritic spine density in dentate granule cells. Society for Neuroscience Abstract Viewer and Itinerary Planner.

Cooke SF, Bliss TVP (2006) Plasticity in the human central nervous system. Brain : a journal of neurology 129:1659–1673.

Cooney JR, Hurlburt JL, Selig DK, Harris KM, Fiala JC (2002) Endosomal compartments serve multiple hippocampal dendritic spines from a widespread rather than a local store of recycling membrane. J Neurosci 22:2215–2224.

Corkin S (1984) Lasting Consequences of Bilateral Medial Temporal Lobectomy: Clinical Course and Experimental Findings in H.M. Seminars in Neurology 4:249–259.

Deller T, Korte M, Chabanis S, Drakew A, Schwegler H, Stefani GG, Zuniga A, Schwarz K, Bonhoeffer T, Zeller R, Frotscher M, Mundel P (2003) Synaptopodin-deficient mice lack a spine apparatus and show deficits in synaptic plasticity. Proc Natl Acad Sci U S A 100:10494–10499.

Desmond NL, Levy WB (1983) Synaptic correlates of associative potentiation/depression: an ultrastructural study in the hippocampus. Brain Research 265:21–30.

Douglas R, Goddard G (1975) Long-term Potentiation of the Perforant Path-Granule Cell Synapse in the Rat Hippocampus. Brain Res 86:205–215.

Doyère V, Srebro B, Laroche S (1997) Heterosynaptic LTD and depotentiation in the medial perforant path of the dentate gyrus in the freely moving rat. J Neurophysiol 77:571–578.

Engert F, Bonhoeffer T (1999) Dendritic spine changes associated with hippocampal long-term synaptic plasticity. Nature 399:66–70.

Fischer, M., Kaech, S., Knutti, D., & Matus, a. (1998). Rapid actin-based plasticity in dendritic spines. Neuron 20(5), 847–54.

Fifkova E, Van Harreveld A, Fifková E (1977) Long-lasting morphological changes in dendritic spines of dentate granular cells following stimulation of the entorhinal area. Journal of Neurocytology 6:211.

Geinisman Y, Berry RW, Disterhoft JF, Power JM, Van der Zee E a (2001) Associative learning elicits the formation of multiple-synapse boutons. J Neurosci 21:5568–5573.

Isaac JT, Nicoll R a, Malenka RC (1995) Evidence for silent synapses: implications for the expression of LTP. Neuron 15:427–434.

Jedlicka P, Vlachos A, Schwarzacher SW, Deller T (2008) A role for the spine apparatus in LTP and spatial learning. Behav Brain Res 192:12–19.

Kemp JM, Powell TP (1971) The structure of the caudate nucleus of the cat: light and electron microscopy. Philosophical transactions of the Royal Society of London Series B, Biological sciences 262:383–401.

Kirov S a, Petrak LJ, Fiala JC, Harris KM (2004) Dendritic spines disappear with chilling but proliferate excessively upon rewarming of mature hippocampus. Neuroscience 127:69–80.

McNaughton BL, Barnes CA (1977) Physiological identification and analysis of dentate granule cell responses to stimulation of the medial and lateral perforant pathways in the rat. The Journal of Comparative Neurology 175:439-453.

Mundel P, Heid HW, Mundel TM, Krüger M, Reiser J, Kriz W (1997) Synaptopodin: an actin-associated protein in telencephalic dendrites and renal podocytes. J Cell Biol 139:193–204.

Nusser Z, Lujan R, Laube G, Roberts JD, Molnar E, Somogyi P (1998) Cell type and pathway dependence of synaptic AMPA receptor number and variability in the hippocampus. Neuron 21:545–559.

Popov VI, Davies H a, Rogachevsky V V, Patrushev I V, Errington ML, Gabbott PL a, Bliss TVP, Stewart MG (2004) Remodelling of synaptic morphology but unchanged synaptic density during late phase long-term potentiation (LTP): a serial section electron micrograph study in the dentate gyrus in the anaesthetised rat. Neuroscience 128:251–262.

Spacek J, Harris KM (1997) Three-dimensional organization of smooth endoplasmic reticulum in hippocampal CA1 dendrites and dendritic spines of the immature and mature rat. J Neurosci 17:190–203.

Treves a, Tashiro a, Witter MP, Moser EI (2008) What is the mammalian dentate gyrus good for? Neuroscience 154:1155–1172.